Extending the span of angular-scanning surface plasmon resonance biosensing: hyphenation, variable-wavelength excitation, and multiplexing

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Design and evaluation of a multiplexed angular-scanning surface plasmon resonance system employing line-laser optics and CCD detection in combination with multi-ligand sensor chips

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ABSTRACT

An angle-scanning Kretschmann configuration surface plasmon resonance (SPR) instrument, allowing multiplexed analysis, is presented. Laser light was guided through optics that converted the collimated light into a line-shaped beam, which was directed to a prism, illuminating the gold sensor surface over a 1x10 mm area. The reflected light was led to a CCD detector providing simultaneous readout of individual analysis spots along the laser line at a selected angle (fixed-angle detection) or in scanning-angle mode (width of 35 degrees). Full SPR curve could be measured every 3.6 s for each illuminated spot on the sensor surface. Two in-house manufactured flow cell designs were used for evaluating multiplexed angular-scanning SPR. The first comprised six parallel channels with the laser line perpendicular to the flow direction in order to allow interrogation of the sensor surface in the six channels. Refractive index changes by varying solution composition, and adsorption of different concentrations of albumin to the sensor surface could be correctly monitored simultaneously in each of the channels. In the second flowcell design the laser line was coinciding with the flow path, allowing recording of SPR curves along a 10-mm length of the sensor surface. Adsorption of layers of positively and negatively charged polyelectrolytes could be consistently measured for sixteen selected positions along the channel. As a proof of principle, several target proteins were immobilized on different positions along the sensor and the binding of various antibodies with these proteins was monitored simultaneously, showing excellent selectivity and reproducibility for probing antibody-protein interactions in a multiplexed fashion.

1. INTRODUCTION

Label-free optical biosensors have found a vast range of applications in life sciences.[1] Among these sensing techniques, surface plasmon resonance (SPR) spectroscopy has extensively been used for real-time assessment of biomolecular interactions. Most conventional SPR setups utilize a Kretschmann configuration comprising a prism coupler with a thin gold layer as a sensor, a He-Ne laser for plasmon excitation, and a photodetector.[2] This allows measurement of shifts in resonance angle caused by analyte binding to ligands immobilized on the sensor surface. SPR instruments mostly comprise only a limited number of flow channels,[3,4] putting constraints on sample throughput. In situations where, for example, libraries of molecules have to be screened for binding against multiple target proteins, higher throughput SPR detection is in demand. The gain in throughput can be achieved by measuring multiple

binding events simultaneously, e.g. by having multiple ligands immobilized on the surface of the SPR sensor and/or by having the possibility to measure more samples in parallel.
Multiplex SPR detection has been achieved with imaging SPR (iSPR).[5, 6] iSPR is usually performed in fixed angle mode with a charge-coupled device (CCD) camera used as a detector, capturing the reflected light modulation on the entire sensorchip.[7, 8] These intensity changes can be monitored for regions of interest (ROIs) in real time through the entire sensor, allowing simultaneous, continuous observation of several interactions (multiplexing) on the surface of sensor chip. These interactions are displayed in a sensorgram at the same time of monitoring. Multiplex SPR detection providing monitoring of angular shifts over a limited range has been introduced, [9-12] employing a scanning mirror directly after the light source, enabling scanning of the incidents angle over 8 degrees. [13-19]
In this study, multiplexed analysis employing an angular-scanning SPR instrument facilitating the simultaneous recording of full SPR curves with a width of 35 degrees for multiple spots and analytes, is developed and explored. For that, hardware modifications were implemented by stretching the narrow laser beam into a uniform light line, and a CCD camera was used for real-time detection of the reflected laser light from multiple positions. In addition, dedicated software was used to convert the measured light intensities of selected areas on the sensor to full SPR curves. For the fluidic part of the system, two new flow cells were designed and produced in-house. One comprises six horizontal channels in order to allow SPR assessment of six different solutions simultaneously and the other flow cell has a single channel. The position of each of the six flow channels or the ROIs in the single channel flow cell is defined by the CCD software, permitting monitoring of reflected-light intensity changes at variable angles for the selected areas.
The performance of the newly designed systems was evaluated. First, the responsiveness to bulk refractive index changes at the sensor surface was checked by monitoring SPR curves of air or solvent solution. Next, the potential of the multiplexed system for probing analyte adsorption to the gold sensor surface was tested by applying different concentrations of the albumin as well as layers of oppositely-charged polyelectrolytes while simultaneously recording SPR curves of ROIs. Detection of protein binding to polyelectrolyte layers on the sensor surface was also investigated, demonstrating the potential of the multiplexed angular-scanning SPR instrument for correctly probing the ROIs. As a final proof of concept, target proteins were immobilized in separated spots in one single channel and the interaction of three antibodies for the target proteins was studied in a multiplexed fashion by SPR.

2. EXPERIMENTAL

2.1. HARDWARE

A schematic of the optical and fluidic design changes made for allowing multiplexed analysis are shown in Figure 1. SPR analyses were performed using an internally adapted multi-parametric SPR Navi 200 instrument from BioNavis Ltd. (Tampere, Finland). The collimated laser beam (670 or 785 nm) was adopted to a line (1 x 10 mm) using line generating optics (Figure 1a). The laser light was directed at variable angle through the glass prism towards the SPR sensor. The intensity of reflected light was monitored using a CCD camera as a detector. An algorithm was developed and implemented in the BioNavis software for rapid processing of the obtained CCD camera images. The algorithm converts the light intensity levels of selected ROIs to an output signal, which is plotted as a function of the incident angle, providing SPR curves for each of the selected ROIs on the sensor chip. A fluidic design

with six horizontal flow channels (Figure 1b) and another with one vertical channel (Figure 1c) were produced fitting the BioNavis setup. For immobilization of 16 spots evenly positioned in line on the sensor surface, a silicon mask (Figure 1d) was produced in-house.

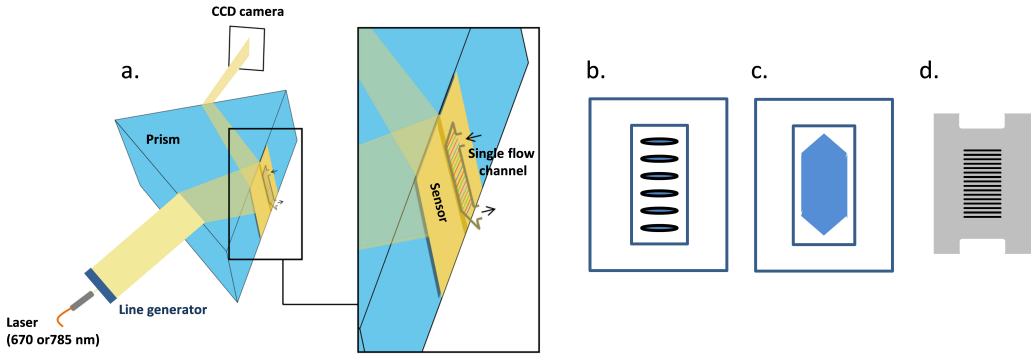

Figure 1. **a.** Schematic of the optical setup for multiplexing SPR. The produced laser line probes the entire length of the surface of the sensor chip covered by the single flow channel. The reflected line is projected on and imaged by the CCD camera detector. The zoomed view of the sensor slide shows the positioning of the single flow cell setup with 16 line-shaped spots on the sensor chip surface. **b.** Six flow-channel format. **c.** Single flow-channel format. Immobilization of multiple compounds on the sensor surface was done online when using the six flow-channel cell or offline when using the single flow-channel cell using the silicon mask.

The flow cells were produced in-house by an advanced DMU/DMC monoBLOCK® series milling machine, (Veenendaal, the Netherlands). Dow Corning 184 PDMS (Dow Corning Europe, Seneffe, Belgium) was used to seal the flow cells and was first degassed in a desiccator. For connection pieces of tubing (IDEX 1581, PEEK, 0.1x 1/32x5, FT, BLUE) were placed at the inlets and outlets of the flow channels. To avoid blockage of the tubing during PDMS backing, core wires were inserted. The tubing was fixed with Loctite M-121HP Hysol Medical Device Epoxy Adhesive (Henkel KGaA, Dusseldorf, Germany). The tubing was secured with micro tights (IDEX F-126HX, 1-PIECE Micro Tight, HEADLESS, 6-3, 1/32 IN, peek, red). The flow cell was then baked in an oven for 120 min at 100 °C followed by cooling to room temperature. The six individual fluidic channels were 5.7 mm in length, 0.6 mm in width, and 1.5 mm in depth. The single flow channel had a width of 2.65 mm, a length of 10.5 mm and a depth of 0.33 mm.

The silicone mask was designed and produced in-house. The dow corning 184 silicone elastomer used for this (Dow Corning Europe, Seneffe, Belgium) was first degassed in a desiccator at 1 mbar for 30 min. The mold was then filled with the degassed dow elastomer and baked in an oven for 120 min at 100 °C. Next, the mask with mold was cooled to room temperature after which the mask was removed from the mold. Finally, the mold was cut to exactly fit the BioNavis sensor holder.

2.2. CHEMICALS AND REAGENTS

Sodium chloride (NaCl), poly sodium 4-styrenesulfonate (PSS) (average molecular weight 70 kDa), poly allylamine hydrochloride (PAH) (average molecular weight 15 kDa), polyethylenimine (PEI) (average molecular weight 25 kDa), 2-(N-morpholino) ethanesulfonic

(MES) monohydrate, human serum albumin (HSA), human hemoglobin, transferrin, cytochrome c from bovine heart, anti-human albumin antibody produced in rabbit, anti-human hemoglobin antibody produced in rabbit, anti-myoglobin antibody produced in rabbit, phosphate buffered saline (PBS), ethanolamine hydrochloride, L-cysteine, *N*-hydroxy succinimide (NHS), *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDC) and sodium hydroxide (NaOH) were purchased from Sigma-Aldrich (Steinheim, Germany). Deionized water was produced by a Milli-Q purification system from Millipore (Amsterdam, the Netherlands). Dulbecco's phosphate-buffered saline (DPBS) was from Gibco, Thermo Fisher Scientific (Massachusetts, USA).

2.3. MULTIPLEXED SPR USING THE SIX-CHANNEL FLOW CELL

An integrated SPR Navi peristaltic pump of the SPR device was used for two of the fluidic channels, whereas a Gilson minipuls-2 L4 peristaltic pump (Middleton, USA) was used for the remaining four channels. The flow rate was 30 µL/min for all channels, and the SPR temperature was set at 24 °C. All measurements were performed with the 785-nm laser in combination with gold (Au) sensor chips (from BioNavis). The system was tested for air, Milli-Q water and ethanol. In addition, HSA (0, 0.03, 0.1, 0.3, 1.0 and 3.0 mg/mL) in Dulbecco's phosphate-buffered saline (DPBS), were analyzed.

2.4. MULTIPLEXED SPR USING THE SINGLE CHANNEL FLOW CELL

All the measurements were performed with the Au sensor chips, using 785-nm laser source.
Online immobilization of polyelectrolytes. The flow rate was 50 µL/min and the flow cell temperature was 20 °C. After obtaining stable baselines for all the ROIs using 0.15 M NaCl as running solvent, the surface was cleaned twice with a 5% Hellmanex solution for 4 min. Polyelectrolytes were deposited successively via plug-injections (4 min each) of solutions containing 0.1 mg/ml PEI, 0.1 mg/ml PSS and 0.1 mg/ml PAH (all in running solution), respectively. After each experiment, the sensor surface was regenerated using a 5% hellmanex solution plug-injection for 4 min.
Offline immobilization of polyelectrolytes. Sensor chip cleaning was performed by pipetting 5% Hellmanex solution over the complete surface of the sensor chip and incubating for 4 min. The sensor chip was then rinsed with 0.15 M NaCl. Next, the sensor chip was incubated with solutions of 0.1 mg/ml PEI followed by 0.1 mg/ml PSS (both in 0.15 M NaCl), each solution for 4 min covering. For exposing only half of the sensor chip to polyelectrolyte, half of the Au surface was covered with tape, followed by pipetting 0.1 mg/ml PAH in 0.15 M NaCl over the non-covered half of the sensor chip. After immobilization, the sensor chip was put in a BioNavis holder and inserted into the SPR instrument. The sensor temperature was set at 20 °C. After obtaining a stable baseline (usually within 2-4 min), analyses were initiated. As a test experiment, 1 mg/mL lysozyme (in 0.15 M NaCl) was plug-injected at a flow rate of 50.

2.5. PROBING PROTEIN-ANTIBODY INTERACTIONS BY MULTIPLEXED SPR

Offline protein immobilization. For protein immobilization, a carboxymethyl dextran (CMD) hydrogel gold sensor chip (BioNavis) was first activated with a solution containing 0.2 M EDC and 0.1 M NHS in water at room temperature for 10 min by pipetting the solution over the entire surface of the sensor chip. The surface was then washed with 5 mM of MES hydrochloride (adjusted to pH 4 with NaOH) and dried under a gentle nitrogen stream. The sensor chip was then positioned in the sensor chip holder for offline immobilization, and the in-house developed silicon mask (Figure 1d) with sixteen slit-shaped openings was tightly placed over the surface of the sensor chip. Immobilization of proteins was done by pipetting their solutions into the individual slits using a 1-µl Agilent syringe needle followed by incubation, deactivation and washing steps standard to EDC activated CMD immobilizations

for SPR.

Antibody-protein binding analysis (direct assay): After protein immobilization, the sensor chip in the holder was placed in the SPR instrument. On the sensor chip, five spots (length, 0.3 mm) of HSA, five hemoglobin, two transferrin, two cytochrome c and two blank spots (reference) were positioned along one line with an inter-spot distance of 0.3 mm. The locations of the immobilized proteins were specified by the software. SPR analyses were done in angular-scan mode using the 785-nm laser. The in-house developed single-channel flow cell was used to direct plug-injections of samples to the spots on the SPR sensor chip. PBS buffer (containing 0.01 M phosphate with 2.7 mM potassium chloride and 0.137 M sodium chloride (pH 7.4)) was used as running buffer at a flow rate of 30 µL/min with a sensor cell temperature of 20 °C. After obtaining a stable baseline, 0.1-100 µg/mL anti-HSA triplicate plug-injections were performed followed by triplicate plug-injections of 0.1-100 µg/mL anti-hemoglobin and then 0.1-100 µg/mL anti-myoglobin antibodies (all diluted in running buffer) for 7 min per plug-injection. For regeneration, a solution of 50 mM NaOH was plug-injected for 1 min. The resonance angle shifts in time for all monitored spots were plotted as sensorgrams. From these data, binding constants for the interactions were calculated with TraceDrawer™ for SPR Navi™ (Ridgeview Instruments AB, Vange, Sweden).

Antibody-protein binding analysis (competitive assay): An antibody or mixed antibodies were pre-incubated with their respective antigens at different concentrations and then analyzed by SPR. Potential cross-reactive and/or non-specific binding was assessed with HSA for hemoglobin and vice versa, and with transferrin, cytochrome c and blank reference spots as negative controls. First, a mixture of 100 µg/ml anti-HSA and 100 µg/ml anti-hemoglobin was incubated on ice for 30 min with 200 µg/ml HSA and 200 µg/ml hemoglobin. Then, the incubated mixtures were plug-injected for 7 min at a flow rate of 30 µl/min. Other analyses involved mixtures of 50 µg/ml anti-HSA and 100 µg/ml anti-hemoglobin pre-incubated with different concentrations of HSA and/or hemoglobin (0-200 µg/ml). From the data obtained, IC50 values were calculated using GraphPad PRISM Software (San Diego, CA, USA).

3. RESULTS AND DISCUSSION

A commercial benchtop angular-scanning SPR instrument was adapted for multiplexed analysis by introducing a line laser for excitation and a CCD camera for detection (Figure 1a). Multiplexing was studied using a six-channel flow cell (figure 1b) and a single-channel flow cell (Figure 1c). Custom-developed software allowed selection of ROIs. For that, the positions of the flow channels and the silicon mask openings were determined by monitoring the reflected light of the laser line over its entire length at the resonance angle for air (~43 degrees). The positions where void flow channels or silicon mask openings are located show SPR (i.e. attenuated intensity), whereas the areas where the gold surface is covered by the flow cell or mask material, no resonance occurs (no change in intensity). After the selection of ROIs, the scanning angle is selected. While performing SPR analysis, the software determines an average signal for the selected ROIs as a function of incident angle, which is then plotted as SPR curves. From these the shifts in the SPR dip angle can be monitored in time, producing a sensorgram.

3.1. BASIC PERFORMANCE OF THE MULTIPLEXED SPR SETUP

First experiments were carried out with the six flow-channel cell using a bare gold sensor surface. In order to check whether the introduced optics worked adequately and ROIs were

selected properly, SPR curves were monitored for each channel filled with air or 0.15 M NaCl in water. The system allowed simultaneous monitoring of full SPR curves from each channel (Figures 2a and 2b), showing similar and correct resonance angles for the respective media (~43.05 degrees for air and ~68.60 for the NaCl solution). Potential performance differences among the channels were evaluated by monitoring the SPR response caused by plugs of ethanol using water as running solvent. The shift in resonance angle caused by the change of the solvent's refractive index was properly monitored for all channels simultaneously, yielding very similar sensorgrams (Figure 2c) and thus showing satisfactory inter-channel consistency. Each channel also exhibited excellent plug-to-plug repeatability (Figure 2c). The multiplexing ability was further evaluated by analysis of HSA in water, plug-injecting different concentrations (0-3.0 mg/mL) into each of the six channels. The resulting sensorgrams nicely show the adsorption of HSA to the gold surface (Figure 2d) with larger shifts observed for higher concentrations of HSA injected. At an injected concentration of 1 mg/ml and higher, the surface was saturated, as the same plateau signal was reached as for the 3.0-mg/mL solution.

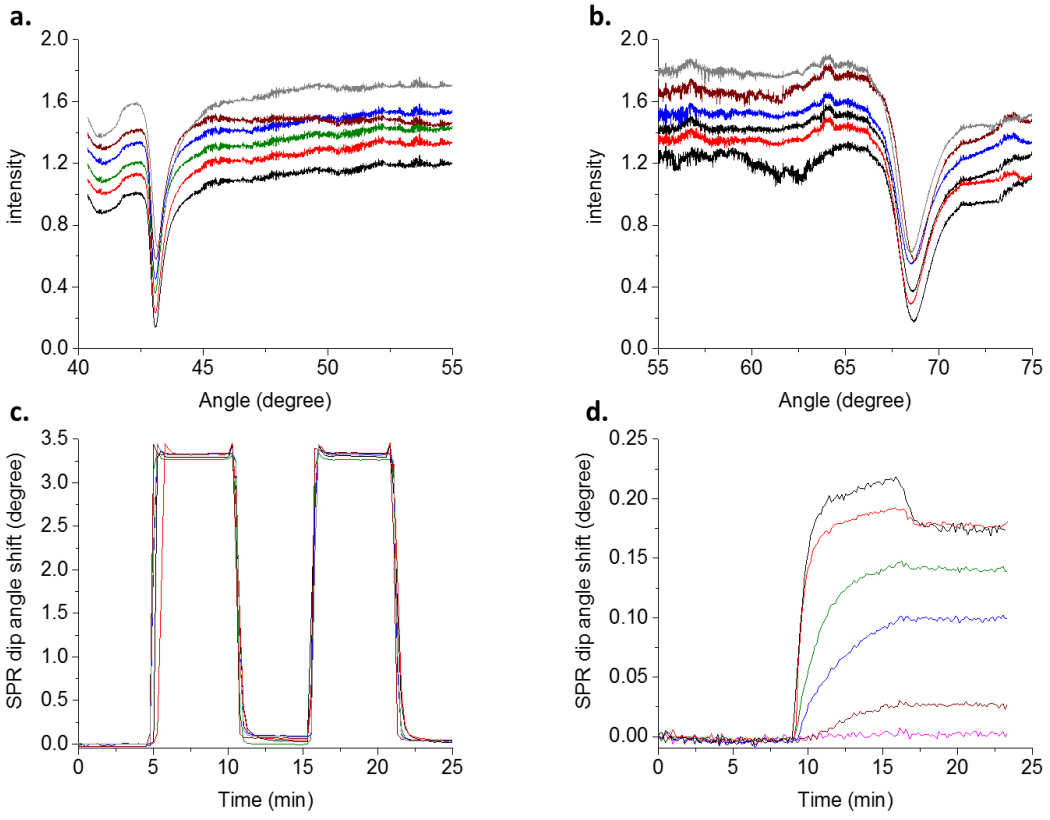

Figure 2. Multiplexed angular scanning SPR using the six-channel flow channel and a bare gold sensor. The figures show the individual responses obtained for each respective channels. Full SPR curves obtained for **a.** air, **b.** water; curves were given an intensity offset for clarity (avoiding overlap). Sensorgrams (resonance angle shift vs. time) obtained for **c.** two consecutive plug-injections of ethanol using water as running liquid, and **d.** plug injections of HSA in water with each channel filled with a different concentration (0, 0.03, 0.1, 0.3, 1.0 and 3.0 mg/mL for channel 1 (pink), 2 (brown), 3 (blue), 4 (green), 5 (red), 6 (black), respectively.

Overall, these results show that the new angular scanning SPR setup with line-laser optics and CCD detector allows simultaneous recording of correct SPR curves and sensorgrams from predefined spatially-separated areas on the sensor surface with good repeatability.

3.2. SINGLE-CHANNEL MULTIPLEXED SPR

Further evaluation of the multiplexing capabilities of the modified SPR instrument was pursued with the single-channel flowcell design for which the laser line was coinciding with the flow path, allowing recording of SPR curves along a 10-mm length of the sensor surface. For that, sixteen positions (length, 0.3 mm; mutual distance, 0.3 mm) equally spaced along the channel were designated by the software for probing SPR signals. Subsequently, the full SPR curves for all sixteen spots of blank gold sensor surface were recorded simultaneously in the liquid media using 0.15 mM NaCl as running solvent. Similar SPR curves exhibiting the same dip angle were obtained for each spot, demonstrating uniform performance. In order to check the responses upon molecular adsorption to the gold surface, solutions of PEI, PSS, and PAH were successively flushed (4 min each) through the channel while recording SPR curves for all sixteen spots. Figure 3a shows the obtained sensorgrams which reflect the consecutive polymer binding events. For all sixteen selected spots measured, similar resonance-angle shifts were observed for each applied layer. The baseline noise in the sensorgrams was 3.7 times higher for the multiplexed instrument as compared to the standard instrument, which probably is caused by the lower absolute intensity of the laser excitation per spot in the multiplexed system.

In order to verify the correct selection of the ROIs utilizing the camera, a PEI-PSS double layer and a PEI-PSS-PAH triple layer were adsorbed to two zones of the sensor surface, respectively, and the electrostatic binding of lysozyme (pI 11; positively charged) was monitored. Eight individual spots were selected for each of the zones using the software. First, a baseline was

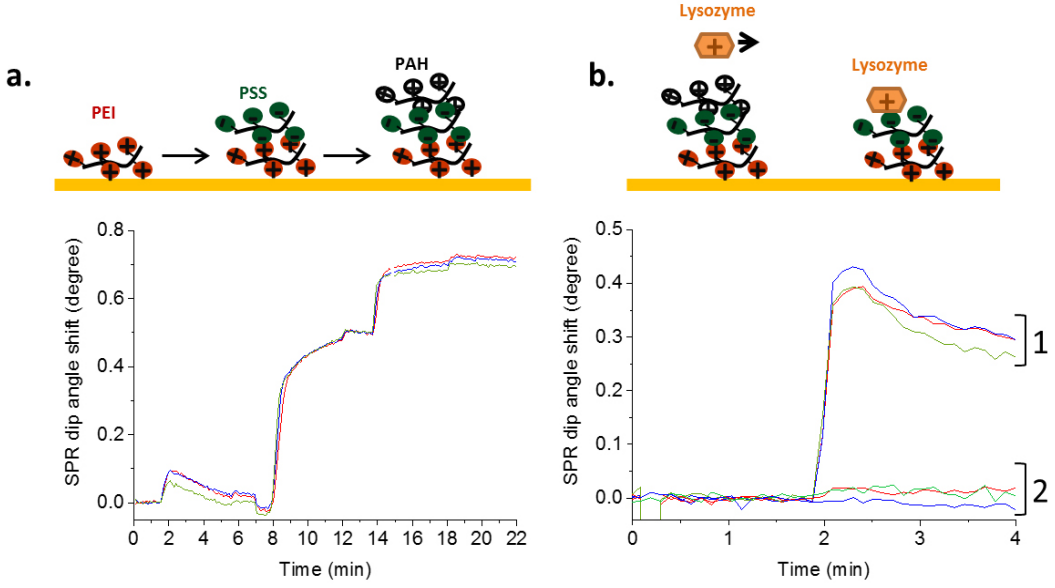

Figure 3. Sensorgrams of **a.** online successive deposition of the polyelectrolytes indicated at the top, and **b.** injections of lysozyme on a sensor chip of which half of the spots comprised a PEI-PSS double layer (negatively charged; region 1) and the other half a PEI-PSS-PAH triple layer (positively charged; region 2).

established for all 16 spots when exposed to 0.15 M NaCl only. Subsequently, a solution of lysozyme (1 mg/mL in 0.15 mM NaCl) was plug-injected for 1 min at 50 µl/min and SPR curves were recorded simultaneously for all spots. The resulting sensorgrams (Figure 3b) recorded for the spots in the first zone (PEI-PSS) show typical protein binding curves, indicating significant interaction of lysozyme with the negatively charged layer. For the spots in the second zone (PEI-PSS-PAH), no change of SPR signal was observed, indicating no binding of lysozyme and confirming the effectiveness of such a triple layer as non-adsorptive coating for positively charged proteins. These results indicate that each spot can be probed correctly by the multiplexed angular-scanning SPR instrument.

3.3. ANTIBODY-PROTEIN INTERACTION ANALYSIS BY MULTIPLEXED ANGULAR-SCANNING SPR

As a proof of principle for more practical SPR analyses, the potential of the multiplexed angular-scanning SPR system for the simultaneous monitoring of multiple biomolecular interactions was investigated. For that, the detection of the selective binding of antibodies with target proteins was used as a model test. Solutions (1 mg/mL each) of the target proteins HSA, hemoglobin, transferrin and cytochrome c in 5 mM MES hydrochloride (pH 4) was deposited in linearly aligned spots (length, 0.3 mm; mutual distance, 0.3 mm) on the surface of a CMD hydrogel gold sensor chip using a silicon mask with sixteen separate slits (see section 2.5). Spots 1-5 were HSA, 6-10 were hemoglobin, 11-12 were blank (no protein applied), 13-14 were transferrin, and 15-16 were cytochrome c. Spots 11 and 12 were used for referencing the signals measured for the other spots (i.e. subtraction of the background signal caused by the sample solvent). The chip with the immobilized proteins was installed in the multiplexed SPR instrument. Figure S1 shows full SPR curves of each spot could be measured simultaneously with the multiplexed system using the single channel flow cell. SPR curves recorded of the spots before protein immobilization (i.e. bare CMD surface) show very similar curves with resonance angles of 66.0 degrees. Specific shifts of the resonance angle were observed for the respective immobilized proteins in the same figure.

Subsequently, using the single-channel flow cell, separate solutions of HSA and hemoglobin antibodies were successively injected (three times anti-HSA followed by three times anti-hemoglobin). Between protein injections, the sensor surface was regenerated with 50 mM

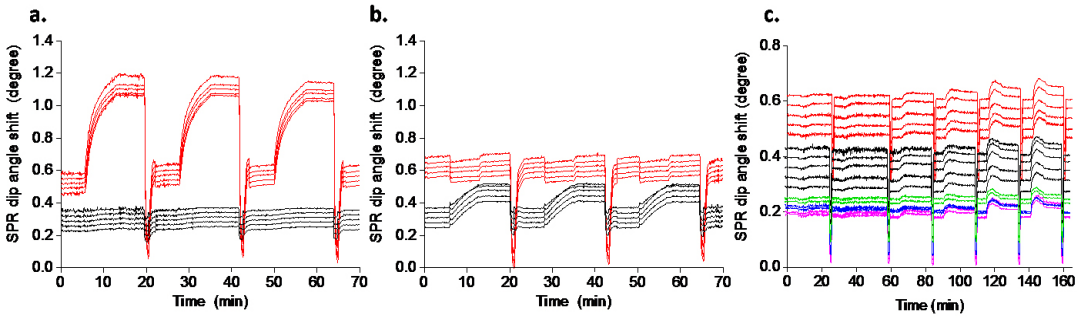

Figure 4. Multiplexed angle-scanning SPR using a sensor with multiple immobilized proteins. Sensorgrams (baseline subtracted) for three consecutive plug-injections of **a.** anti-HSA and **b.** anti-hemoglobin. **c.** Sensorgrams (not baseline subtracted) for six consecutive plug-injections of anti-myoglobin at increasing concentration (0.1-100 µg/mL). Immobilized proteins: HSA (five spots; red lines), hemoglobin (five spots; black lines), transferrin (two spots; green lines), cytochrome c (two spots; blue lines), no protein (two spots; purple lines). Sensorgrams were given an intensity offset for clarity (avoiding overlap).

NaOH for 1 min. SPR curves were continuously monitored for all spots. The sensorgrams obtained for the spots upon anti-HSA and anti-hemoglobin are shown in Figure 4a and 4b, respectively (for raw data, see Figure S2). Upon anti-HSA injection the five spots with immobilized HSA uniformly showed intense SPR responses, whereas no binding of anti-HSA was observed for the spots with immobilized hemoglobin, transferrin and cytochrome c (for transferrin and cytochrome c, see Figure S2), indicating proper selectivity. For the hemoglobin-antibody injections, only specific binding was observed for the five hemoglobin spots which showed very similar sensorgrams. As a negative control, anti-myoglobin was sequentially injected in concentrations increasing from 0.1 to 100 µg/ml (six injections; Figure 4c). Upon injection of the high concentration antibody, temporary increase of the signal was observed as a result of the bulk effect which in principle can be corrected with the reference channels signal (purple lines). Importantly, the simultaneously monitored sensorgrams showed no specific binding to any of the immobilized proteins, nicely confirming the suitability of the multiplexed SPR approach.

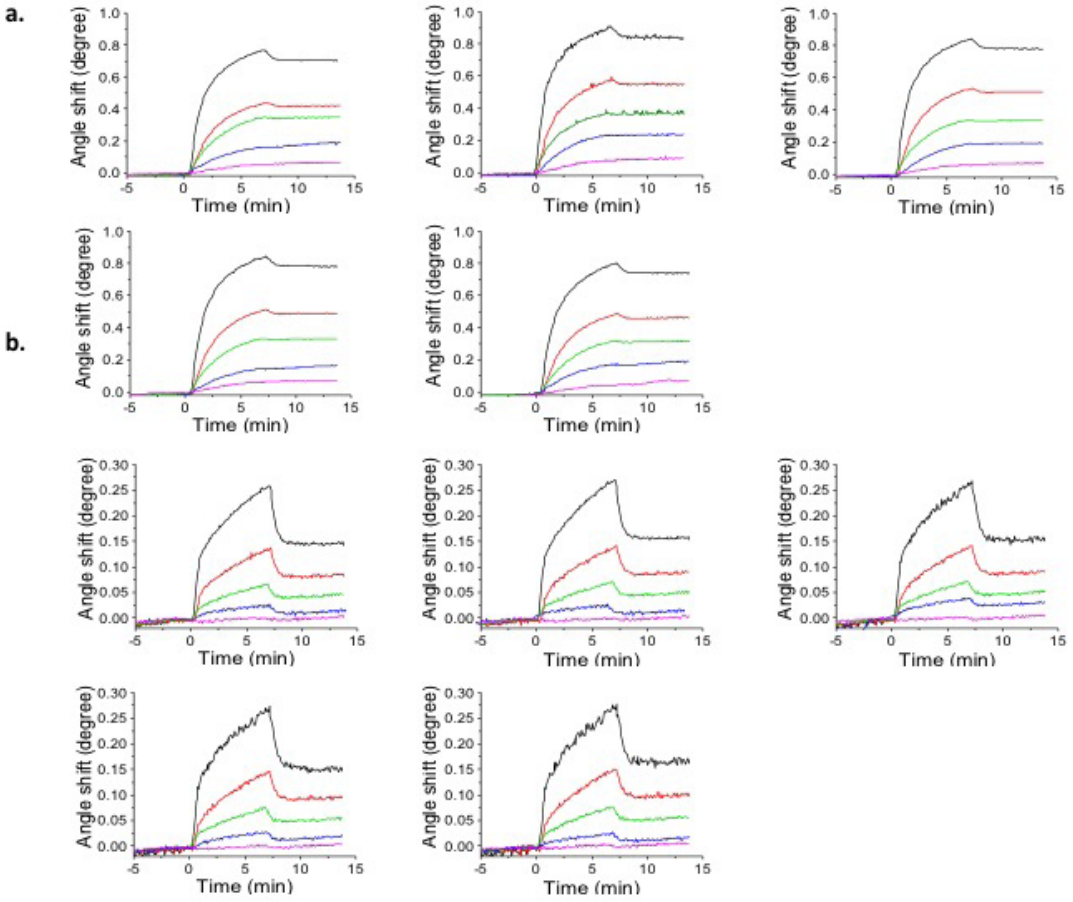

Figure 5. Multiplexed angle-scanning SPR using a sensor with multiple immobilized proteins. Sensorgrams for **a.** the five HSA spots upon exposure to increasing concentrations of anti-HSA, and **b.** the five hemoglobin spots upon exposure to increasing concentrations of anti-hemoglobin. Antibody concentrations: 1 (purple), 10 (blue), 25 (green), 50 (red) and 100 (black) µg/ml.

In order to study the potential of the multiplexed angular-scanning setup for measuring antibody-ligand binding characteristics, different concentrations of anti-HSA and anti-hemoglobin (0.1-100 µg/ml) were plug-injected in triplicate and sensorgrams were constructed from the SPR curves obtained for the HSA and hemoglobin spots (Figures 5a and 5b, respectively). The figures show consistent performance of the various spots. For example, for 50 µg/ml anti-HSA, the average SDs within one spot and in between spots were 0.019 and 0.018 degrees, respectively. For 50 µg/ml anti-hemoglobin, the average SDs within one spot and in between spots were 0.004 and 0.011 degrees, respectively.

From the sensorgrams obtained, the association rate (k_a), dissociation rate (k_d) and dissociation (K_D) constants for the antibody-protein interactions were calculated using the bivalent interaction model fitting in TraceDrawer software (Table S1). The kinetics calculation showed higher association rate and slower dissociation rate of the HSA antibody to HSA protein in comparison with the Hemoglobin antibody to hemoglobin protein. The K_Ds obtained for the HSA-anti-HSA complex from the respective spots under the here introduced experimental conditions, were 34.1±2.3 nM, 50.9±2.7 nM, 55.1±3.4 nM, 58.8±4.2 nM and 61.7±3.8 nM. For hemoglobin, the obtained K_Ds were 21.2±2.8 µM, 43.7±3.0 µM, 44.3±5.3 µM, 38.8±3.0 µM and 56.7±4.8 µM. The obtained K_Ds show good repeatability within each spot and are similar for each antibody. Lower K_D values indicate higher binding affinity of the HSA antibody to HSA protein, compare to hemoglobin antibody to hemoglobin protein.

In order to demonstrate the further applicability of the multiplexed angular-scanning SPR system for protein binding assays, antibodies were pre-incubated with their respective antigens and analyzed. A mixture of anti-hemoglobin and anti-HSA was plug-injected in the presence of increasing concentrations of HSA and hemoglobin and full SPR curves were recorded simultaneously for all immobilized protein spots. The binding events of high antibody concentrations with and without pre-incubation of antigenic proteins led to the sensorgram shown in Figure S3. Sensorgrams of pre-incubation of antibodies with different concentrations of proteins for the HSA and hemoglobin positions are shown in Figure 6a and Figure 6b, respectively. SPR clearly showed a decrease of antibody binding with increasing concentrations of antigenic protein in the injected incubation mixture. Results showed similar curve shape and affinity in between the spots, which confirms the reproducibility of the system in between spots for biomolecular interaction analysis. From the obtained sensorgrams for each respective spot, the IC50 values for the antigens were determined, yielding 155±3 nM, 143±15 nM, 157±23 nM, 145±11 nM and 156±24 nM for HSA, and 73.1±2.4 nM, 75.3±1.0 nM, 75.3±1.3 nM, 75.0±2.1 nM and 74.1±1.5 nM for hemoglobin.

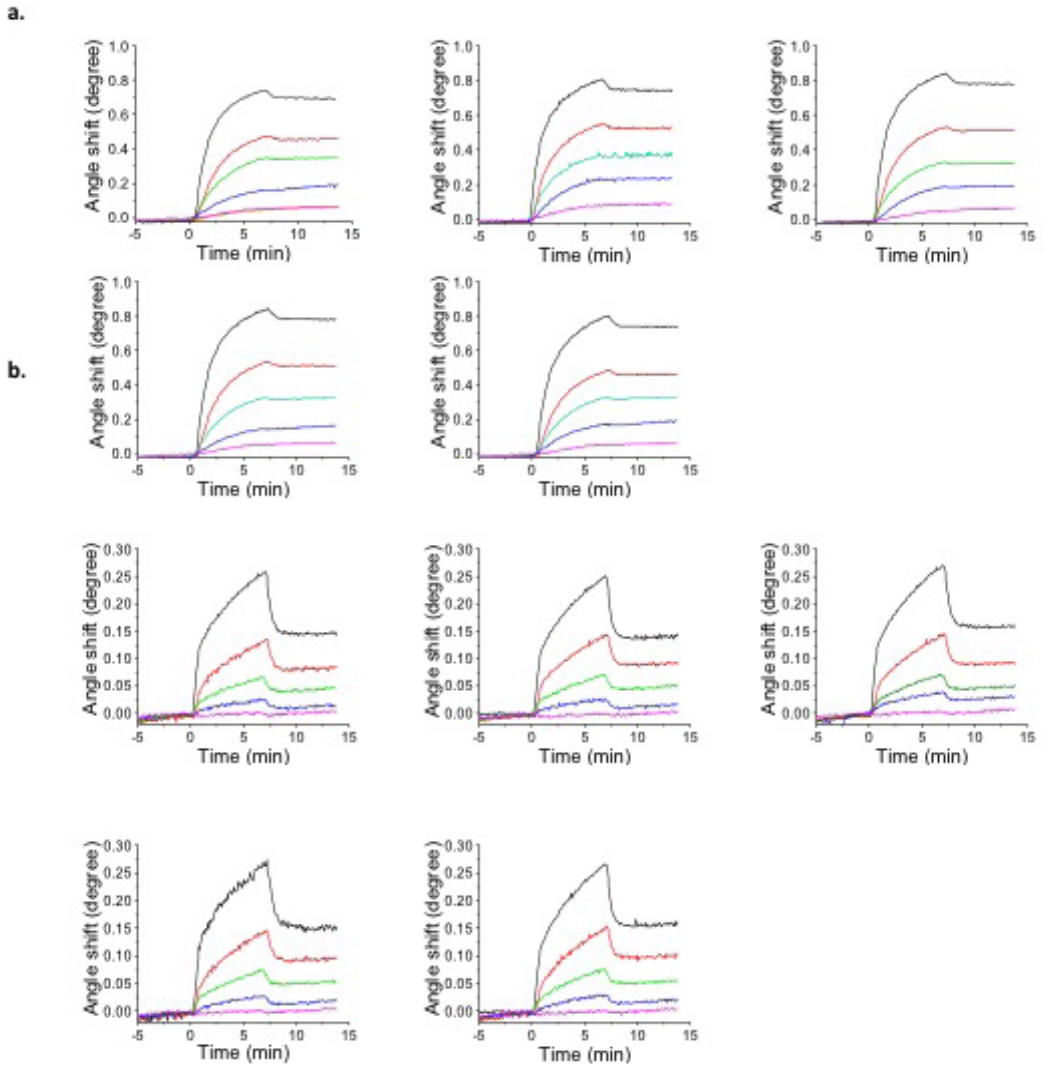

Figure 6. Multiplexed angle-scanning SPR using a sensor with multiple immobilized proteins. Sensorgrams for **a.** the five HSA spots upon exposure to anti-HSA (200 µg/mL) incubated with different concentrations of HSA, and **b.** the five hemoglobin spots upon exposure to increasing anti-hemoglobin antibody (200 µg/mL) incubated with different concentrations of hemoglobin. Protein-ligand concentrations: 100 (purple), 50 (blue), 25 (green), 10 (red) and 1 (black) µg/mL.

4. CONCLUSION

This study presents the development of a multiplexed line-laser based angular-scanning SPR system with a Kretschmann configuration allowing binding evaluation and measurement of multiple analytes and/or ligands simultaneously. Continuous angular scanning between 40-75 degrees provided full SPR curves for the complete sensor area covered by the laser line. This wide angular range permits SPR measurements in both air and liquid media. Flow cells with six parallel channels or a single channel with up to sixteen detection spots were utilized for multiplexed analysis. The basic performance of the new setup was tested monitoring responses by ethanol refractive index changes, protein adsorption and polyelectrolyte-layer adsorption. The results showed the capability of the newly developed system for performing reliable multiplex analyses. Applicability for biosensing was shown by multiplexed measuring antibody-antigenic protein interactions. Several proteins were immobilized in up to sixteen discrete spots in line on the surface of a CMD hydrogel gold sensor chip. Analysis of (mixtures of) antibodies provided a simultaneous assessment of binding to each of the immobilized antigens, showing excellent selectivity, repeatability and reproducibility among the probed spots. Obtained full SPR curves and sensorgrams allowed calculation of binding-kinetic and inhibition constants (k_a, k_d, $K_{D,}$ IC50). The here presented system substantially increases the throughput of full angle scanning SPR in single flow channel format. In theory, the presented system will allow extension of multiplexed SPR detection of approximately up to 100 ROIs (20-pixel lines per ROI). For that, advanced offline techniques for accurate ligand immobilization on the surface of SPR sensor should be used.

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APPENDIX A. SUPPLEMENTARY INFORMATION

Supporting figures, Figure S1-S3; Table S1

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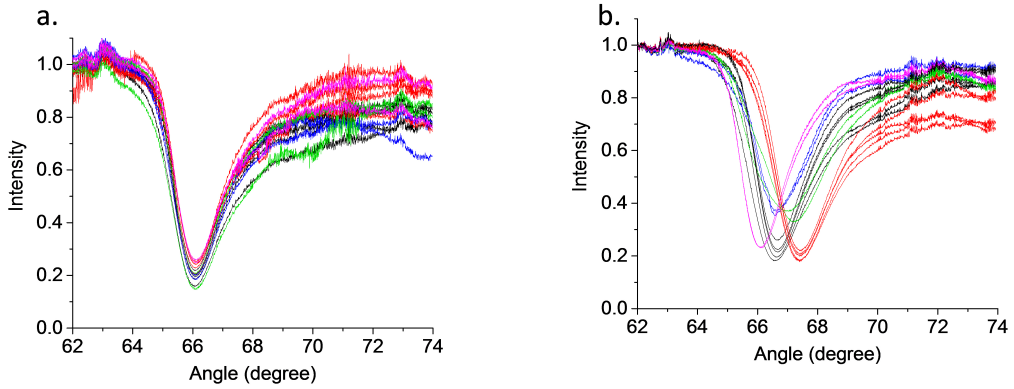

Figure S1. Full SPR curves of sixteen spots measured simultaneously using the multiplexed angle-scanning instrument with the single-channel flow cell and CMD sensor chip. SPR curves recorded for each spot **a.** before protein immobilization and **b.** after protein immobilization. Immobilized proteins: HSA (five spots; red lines), hemoglobin (five spots; black lines), transferrin (two spots; green lines), cytochrome c (two spots; blue lines), no protein (two spots; purple lines).

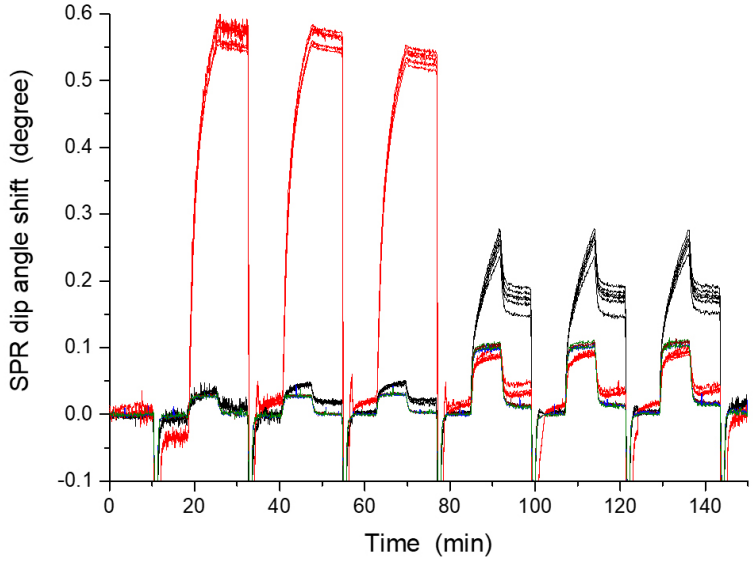

Figure S2. Multiplexed angle-scanning SPR using a sensor with multiple immobilized proteins. Sensorgrams (not baseline subtracted) for three consecutive plug-injections of 50 µg/ml anti-HSA and three injections of 100 µg/ml anti-hemoglobin. Immobilized proteins: HSA (five spots; red lines), hemoglobin (five spots; black lines), transferrin (two spots; green lines), cytochrome c (two spots; blue lines), no protein (two spots; purple lines).

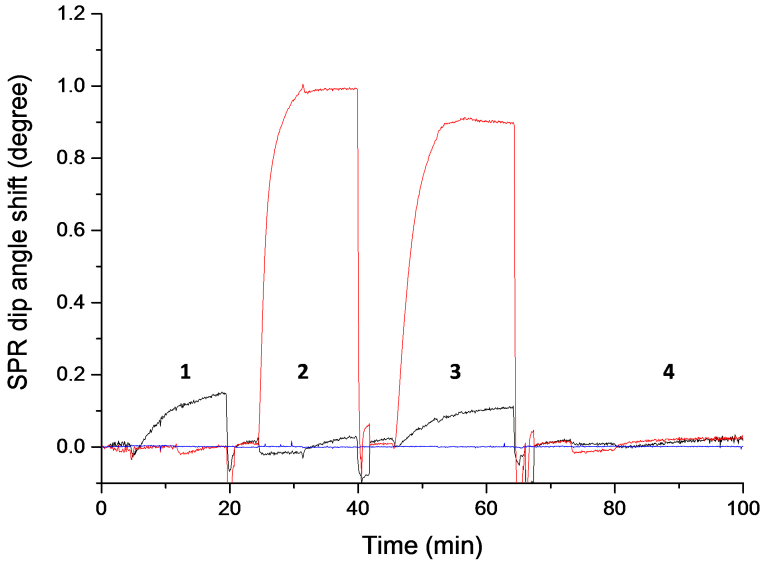

Figure S3. Sensorgrams (baseline subtracted) for 100 µg/ml of **1.** anti-hemoglobin antibody, **2.** anti-HSA, **3.** a mixture of anti-hemoglobin and anti-HSA, and **4.** the antibody mixture pre-incubated with their protein antigens (200 µg/ml). The binding of each of the antibodies was shown to be specific to their immobilized antigen target on the surface of the SPR sensor chip. The full binding of the antigenic proteins in-solution to their respective antibodies resulted in preventing the antibodies from binding to the sensor chip spots. This resulted in observing only the bulk effect, which was corrected for with the sensorgrams obtained from the reference channels.

Table S1. The association rate (k_a), dissociation rate (k_d) and equilibrium dissociation (K_D) constants of the HSA and hemoglobin antibodies with measured IC50 values for HSA and hemoglobin, for each of the five spots per protein calculated from triplicate measurements.

Anti-HSA sample	1	2	3	4	5
k_a $(M^*s)^{-1}$	$5.85\pm0.65 \times 10^3$	$4.56\pm0.30 \times 10^3$	$2.94\pm0.26 \times 10^3$	$2.20\pm0.48 \times 10^3$	$3,83\pm0.83 \times 10^3$
k_d $(s)^{-1}$	$2.00\pm0.19 \times 10^{-4}$	$2.32\pm0.09 \times 10^{-4}$	$1.62\pm0.09 \times 10^{-4}$	$1.71\pm0.20 \times 10^{-4}$	$2,36\pm0.30 \times 10^{-4}$
K_D (M)	$3.41\pm0.23 \times 10^{-8}$	$5.09\pm0.27 \times 10^{-8}$	$5.51\pm0.34 \times 10^{-8}$	$5.88\pm0.42 \times 10^{-8}$	$6,17\pm0.38 \times 10^{-8}$
IC50 (M antigen)	$1.55\pm0.03 \times 10^{-7}$	$1.43\pm0.15 \times 10^{-7}$	$1.57\pm0.23 \times 10^{-7}$	$1.45\pm0.11 \times 10^{-7}$	$1.56\pm0.24 \times 10^{-7}$

Anti-Hemoglobin sample	1	2	3	4	5
k_a $(M^*s)^{-1}$	$1.34 \pm0.84 \times 10^3$	$5.23\pm0.44 \times 10^2$	$5.68 \pm0.51 \times 10^3$	$7.74\pm0.36 \times 10^2$	$5.28\pm0.42 \times 10^2$
k_d $(s)^{-1}$	$2.84\pm0.25 \times 10^{-2}$	$2.29\pm0.13 \times 10^{-2}$	$2.52\pm0.27 \times 10^{-2}$	$3.01\pm0.10 \times 10^{-2}$	$2.92\pm0.21 \times 10^{-2}$
K_D (M)	$2.12\pm0.28 \times 10^{-5.}$	$4.37\pm0.30 \times 10^{-5}$	$4.43\pm0.53 \times 10^{-5}$	$3.88\pm0.30 \times 10^{-5}$	$5.67\pm0.48 \times 10^{-5}$
IC50 (M antigen)	$7.31\pm0.24 \times 10^{-8}$	$7.53\pm0.10 \times 10^{-8}$	$7.53\pm0.13 \times 10^{-8}$	$7.50\pm0.21 \times 10^{-8}$	$7.41\pm0.15 \times 10^{-8}$

06

Summary, discussion and future perspectives

6.1. SUMMARY

Surface plasmon resonance (SPR) is an optical sensing technique through which real-time binding of analytes to their respective ligands, immobilized on a surface (i.e. the sensor), can be sensitively monitored. This thesis describes method development and hardware improvements, which significantly expand the possibilities of angular-scanning SPR instrumentation. The newly developed SPR methods encompass innovations which enable:

I. Determination of the affinity of individual binders present in one sample by hyphenation of angular-scanning SPR with liquid chromatography (LC);
II. Variable-wavelength angular-scanning SPR allowing selection of the optimum wavelength with respect to properties of the metal layer of the sensor chip, sample matrix, and biomolecular interaction of interest;
III. Multiplexing angular-scanning SPR for parallel and multi-ligand affinity screening.

Despite extensive advances in SPR sensor surface optimization and modification for affinity analysis, SPR is not able to discriminate different ligand-binding components present in one sample. **Chapter 2** describes the coupling of liquid chromatography (LC) to angular-scanning SPR, allowing assessment of the affinity of individual separated components in a sample with the immobilized ligand on the surface of SPR sensor. Size exclusion chromatography with an aqueous mobile phase was used as an SPR-compatible method for size-based separation of proteins. To evaluate the SEC-SPR system, papain-digested antibody, comprising several protein fragments, was prepared as a test sample. The system was optimized for several chromatographic parameters such as injection volume, flow rate, and sample concentration prior to SPR affinity analyses. Online-regeneration of the SPR sensor chip in between injection and potential chromatographic heart-cutting experiments for SPR affinity detection of individual sample components were utilized by implementation of two automated switch valves in the system. The performance evaluation of SEC-SPR system was done by analysis of digested proteins sampled at different incubation time points. Specific label-free SPR analysis of real-time interactions of eluting antibody sample constituents towards their antigenic target was demonstrated.
LC-SPR was further evaluated in **Chapter 3** for affinity monitoring of binding components in pharmaceutical protein samples. The monoclonal antibody (mAb) trastuzumab and its antibody-drug conjugate (ADC), trastuzumab-emtansine (T-DM1), were used as test compounds. These drugs are used for treatment of HER-2 positive breast cancer targeting the HER2 receptor. Size and charge variants potentially present in these sample as result of production modifications and storage conditions may alter drug efficacy. LC-SPR could be used for affinity evaluation of size and charge variants after their separation under near-native

conditions. SEC-SPR of the test mAb and ADC before and after exposure to aggregate-inducing conditions was performed using a sensor with HER2 receptor immobilized on the sensor surface. The ADC showed accelerated formation of aggregation, most probably as a result of drug conjugation to the mAb increasing intermolecular hydrophobic interactions. SPR detection of the size variants indicated that the monomer and aggregates of the mAb and ADC have similar affinity toward the HER2 receptor. Cation exchange chromatography (CEX) under native conditions was coupled to SPR to monitor the affinity of charge variants present in the antibody sample. No difference in the ligand binding capacity (R_{max}) of the separated trastuzumab charge variants was observed using CEX-SPR. In order to allow protein variant assignment, parallel MS detection was added to the SEC-SPR setup using a column effluent split. The feasibility of the SEC-SPR/MS system was demonstrated by the analysis of trastuzumab and T-DM1 providing assignment of antibody glycoforms and/or determination of the drug-to-antibody ratio (DAR), while simultaneously monitoring HER2 receptor affinity of the separated species.

Next to incorporation of SPR as an analytical detector for LC, some technical advances in standalone SPR optical sensing were pursued in this thesis. **Chapter 4** presents the development of variable-wavelength angular-scanning SPR employing Kretschmann configuration by the adding and replacing optical arts of a multi-parametric BioNavis SPR device. With the proposed set-up, an optimum wavelength with respect to the sensorchip metal layer, sample matrix and biomolecular interaction of interest can be selected. The evaluation of the system was performed by recording full SPR curves at different wavelengths ranging from 600 to 890 nm using sensor surfaces of silver, gold and gold with deposited silicon oxide, aluminum oxide, titanium oxide and indium tin oxide, which were exposed to air and an aqueous solution of sodium chloride. The results showed wavelength dependencies of the SPR dip position and width of various sensor materials. The system capabilities for monitoring molecular binding was further investigated by layer-by-layer adsorption of charged polyelectrolytes at 600, 670, 785, and 890 nm in angular scanning mode. Although shorter wavelength induced larger angular shifts as result of layer deposition, longer wavelength also showed sharper SPR dip curves and higher signal-to-noise ratios. Since the SPR dip angle position occurred at lower angle using longer wavelength, the angular detection window for liquid solutions is larger for longer wavelengths. To demonstrate the system applicability for protein binding events, antibody interactions with immobilized target protein were probed. The results showed that antibody-protein binding detection is possible down to 0.6 nM using excitation light of 890 nm. The presented setup in this chapter allows continuous recording of full SPR curves in time at any selected wavelength in the 600–890 nm range using a sensor material of choice.

High-throughput label-free SPR analysis for measuring multiple binding events simultaneously (multiplexing) and consequently reducing analysis times is commonly performed using imaging SPR (iSPR). Most commercially available iSPR are measuring the light intensity changes at fixed angles and/or by monitoring angle shifts over a short angle range (max. 8 degrees) in liquid solution using a CCD camera as a detector. Hardware and software development towards a multiplexing angle-scanning (39-78 degree) Kretschmann configuration SPR instrument is presented in **Chapter 5**. Line excitation of a 1x10 mm sensor surface was achieved by directing the laser beam through line generating optics. A CCD camera was used for monitoring the reflected line laser light. The developed data processing software allowed selection of number, shape and position of individual analysis spots (regions of interest (ROIs)). The proposed setup enables intensity measurement of the reflected light at a fixed angle or monitoring SPR angle shifts from fully recorded SPR curves in angular scanning mode. Two new fluidic channel flow cell designs were produced in-house. The first design consists of six parallel flow channels perpendicular to the laser

line, enabling simultaneous monitoring of six solutions on the SPR sensor. The second design, consists of one flow channel in line with the laser line covering the entire length of the sensor, allowing the simultaneous measurement of the interaction of one analyte with multiple immobilized ligands on the surface of the SPR sensor. The developed system was first evaluated by monitoring refractive index changes of solutions, using the six-channel flow cell, showing uniform responses and SPR dip angle shifts for the different channels. The correct selection of the flow cell positions by the camera software was confirmed by measuring the adsorption of albumin at different concentrations on a bare gold sensor employing the six-channel flow cell. Layer-by-layer adsorption of charged polyelectrolytes was used for evaluation of the readout of the single-channel cell. The possibility of applying and monitoring different ROIs was evaluated by creating positively and half negatively charged regions on the sensor, and probing the interaction of an overall positively charged protein with the sensor surface. Protein adsorption on the negatively charged surface part could be measured effectively, while no SPR signal was obtained for the positive region. The ultimate performance of the developed multiplex scanning-angle SPR system was assessed by monitoring the direct and competitive interaction of antibody analytes with up to 16 protein spots that were immobilized in one line on the sensor surface. Full SPR curves of the 16 spots could be measured simultaneously probing various antibody-protein interactions at the same time.

6.2. DISCUSSION AND FUTURE PERSPECTIVES

This thesis describes the development of the hyphenation of LC separation techniques to SPR and hardware developments for angle scanning SPR, introducing multi-wavelength SPR excitation and multiplexing SPR detection. Practical considerations and future developments are briefly described in this section.

6.2.1. HYPHENATION OF LC AND SPR

Hyphenation of LC to SPR allows separation of multiple binding components in a sample prior to monitoring their affinity toward the immobilized ligand on the sensor. SPR ligand-analyte affinity monitoring is only possible when these ligand-analyte proteins are in their active (commonly native) state. For this reason, preservation of analytes and ligands close to their native state is an essential factor when selecting suitable separation techniques prior to SPR. For example, LC separation involving eluents containing organic solvents are excluded, since organic solvents cause protein denaturation and consequently loss of biological activity. Like shown in this thesis, SEC and CEX can be used, but other protein separation techniques, such as hydrophobic interaction chromatography (HIC)[1] or field-flow fractionation (FFF)[2] could be potential candidates to be combined with SPR. HIC utilizes descending salt gradients for moderating hydrophobic interactions of the analyte proteins with the stationary phase, and, in contrast to reversed-phase LC does not employ organic solvents.[3] Consequently, during HIC separations the biological activity of analytes is maintained. In FFF, separation of large molecules is performed in an open channel, i.e., without use of a stationary phase and protein conformations are not affected by high ionic strengths or organic solvents. In addition, mechanical or shear stress on proteins is minimal.[4,5] Affinity chromatography[6] prior to SPR analysis could be utilized as a purification step when binders with multiple binding sites are present. For example, in the model system introduced in Chapter 2, digested antibodies with Fc binding sites can be purified prior to

monitoring the affinity of Fab fragments with the immobilized ligand on the surface of SPR. Higher resolution separation could be obtained by utilizing multidimensional native LC separation techniques.[7] Monitoring the SPR affinity of components separated based on their size by a first dimension (e.g. with SEC) and charge in the second dimension (e.g. with CEX), would be an interesting option. The use of a trapping method with solvent switching and analyte focusing between the dimensions or prior to SPR could be interesting options to study, taking e.g. the work of Van de Ven et al.[8] and Gargano et al.[9] as inspiration.
Capillary electrophoresis (CE) can be considered a powerful native protein separation technique, as high-resolution separation can be obtained under very mild buffer conditions without using of organic solvents. However, due to the small capillary and injection volumes (µl and nl range, respectively), coupling of CE with SPR is challenging. Recently, hyphenation of CE to SPR was reported by Domínguez-Vega et al.[10]. Using a dedicated microfluidic flow cell affinity assessment of protein mixtures (incl. protein variants) was achieved. Nonetheless, the technical issues related to low-volume SPR cells and sensor grounding to avoid SPR detection disturbance by the CE voltage, makes, at least for the moment, LC-SPR a more practical option for future routine analysis.
On-line SPR evaluation of binding kinetics and affinity parameters of separated sample components, poses some issues related to actual concentration, mass transfer limitation, diffusion coefficients and dynamic concentration profiles (peaks). Proper software is required to extract correct absolute affinity data from LC-SPR results.
In Chapter 3, it is shown that LC-SPR can be carried out with parallel MS detection. In this respect, the selection of the appropriate volatile mobile phase which is compatible with both SPR and MS detection, is a crucial aspect. Combining binding and mass information on individual sample components obviously provides a very powerful tool for selective affinity evaluation of samples of unknown composition.

6.2.2. VARIABLE WAVELENGTH ANGULAR-SCANNING SPR

Variable wavelength angular-scanning SPR was developed and explored in order to allow selection of a wavelength most appropriate for e.g. the applied sensor material or ligand-analyte properties, while still have the possibility to determine the SPR dip angle position from full SPR curves. In the system introduced in this thesis, the wavelength selection had to be performed manually and SPR analyses could be performed using only one wavelength at the time. A simple adaption of the proposed system should allow for fast automated wavelength selection, so that, for example, full SPR curves can be recorded at different wavelengths using wavelength programming. It is not difficult to envision the possibility of three-dimensional SPR measurement (i.e. angle-wavelength-intensity), allowing fast selection of the optimal wavelength for specific experiments, prior to the actual affinity analyses. Additionally, such instrument could be used for 'wavelength-scanning' SPR, monitoring the optimal SPR wavelength as a function of changes in the refractive index at the sensor surface. [11, 12]

6.2.3. MULTIPLEXING ANGULAR-SCANNING SPR

Multiplex angular-scanning SPR application for simultaneous detection of several analytes was presented in this thesis. Considering the resolution of the optical setup used, in principle, the presented system would allow multiplexed SPR detection of up to 100 ROI areas (20-pixel lines per ROI), providing good opportunities for performing high-throughput angular-scanning SPR. For that, high-resolution immobilization techniques for applying micro-spots on the sensor surface should be utilized. In addition, dedicated recording and data analysis software should be designed for the handling of the multiple-parameter SPR measurements.

Potentially, the multiplex angular-scanning SPR instrument employing the multiple flow cell configuration might also show useful in the hyphenation of with SPR. During one LC run, multiple heart-cutted peaks could each be led to an individual flow channel with fresh sensor surface. This would avoid overloading/saturation of the SPR sensor by main sample compounds and reduce overall LC-SPR analysis time and sample consumption. Ultimately, integration of multiplexing, variable wavelength and angular scanning SPR in one single system in combination with LC would provide a highly versatile tool for affinity profiling of complex mixtures.

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List of Publications

LIST OF PUBLICATIONS

1. Lakayan, Dina, et al. "On-line coupling of surface plasmon resonance optical sensing to size-exclusion chromatography for affinity assessment of antibody samples." *Journal of Chromatography A* 1452 (2016): 81-88.

2. Lakayan, Dina, et al. "Affinity profiling of monoclonal antibody and antibody-drug-conjugate preparations by coupled liquid chromatography-surface plasmon resonance biosensing." *Analytical and Bioanalytical Chemistry* (2018): 1-12.

3. Lakayan, Dina, et al. "Angular scanning and variable wavelength surface plasmon resonance allowing free sensor surface selection for optimum material-and bio-sensing." *Sensors and Actuators B: Chemical* 259 (2018): 972-979.

4. Lakayan, Dina, et al. "Design and evaluation of a multiplexed angular-scanning surface plasmon resonance system employing line- laser optics and CCD detection in combination with multi-ligand sensor chips." Under review with *Sensors and Actuators B: Chemical*.

Final Words

I started writing down my daily achievements when I was young. These achievements did not necessarily have to be great but were often rather small and insignificant. Today I am at the end of a journey toward one of the most significant achievements of my life. Back then I could never imagine standing where I am at the moment. It has been a long journey and definitely not one that I could have done alone. It would never have been possible without the help and support of my promotor and co-promotor, colleagues, friends, family and loved ones who inspired and guided me along the way.

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EXTENDING THE SPAN OF ANGULAR-SCANNING SURFACE PLASMON RESONANCE BIOSENSING: HYPHENATION, VARIABLE-WAVELENGTH EXCITATION, AND MULTIPLEXING